

METHOD

Field of the Invention

The present invention relates to monitoring polynucleotide amplification reactions.

5 Background to the Invention

The ability to generate multiple copies of a particular polynucleotide in an amplification reaction is important in many commonplace biotechnology processes. The polymerase chain reaction (PCR), as disclosed in US 4,683,202, permits exponential amplification of a polynucleotide to achieve 10 large quantities of the polynucleotide. In its simplest form, PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridise to opposite strands and flank a region of interest in the target DNA. A repetitive series of reaction steps involving template denaturation, primer annealing, and the extension of the annealed 15 primers by DNA polymerase results in the exponential accumulation of the target polynucleotide.

At the start of a PCR reaction, reagents are in excess and the template and product are at a sufficiently low concentration so that product denaturation does not compete with primer binding, and the amplification reaction proceeds 20 at a constant exponential rate.

There are many diagnostic assays that utilise PCR and rely on the quantification of the amplified products. For accuracy and precision, it is necessary to collect quantitative data at a point at which the sample is in the exponential phase of amplification (this is important as it is the exponential 25 phase that provides reproducible results). The need to monitor the amplification reaction can slow down the time taken to complete the diagnostic assay due to the need to take samples of the amplification products and determine the quantity of amplified products present at different time stages.

Real-time (PCR) automates this labourious process by quantitating 30 reaction products for each sample in every amplification cycle. This reaction relies upon the detection and quantification of a fluorescent reporter molecule,

the signal of which increases in direct proportion to the amount of amplified product in a reaction.

There are now many commercially available real-time PCR kits which rely on particular fluorescent molecules.

5 Although real-time PCR has increased sensitivity over traditional (end point) PCR, there are a number of inherent difficulties relating to the use of this system. The disadvantages are due primarily to the use of the fluorescent labels required in real-time PCR techniques. The fluorescent labels must be highly chemically stable, both in terms of the amount of excitation light they can absorb
10 and their ability to withstand the temperature required in the PCR process. The use of fluorescent labels also limits the polynucleotide sequence that may be used as a probe for attachment to the amplified product, as guanine residues are known to act as quenchers in a fluorescent resonant energy transfer process and therefore care must be taken when selecting the sequences immediately
15 adjacent to the position at which the fluorescent molecule is attached to the amplified product.

The limited number of fluorescent molecules available and the common use of a monochromatic energising light source also limits the extent to which multiplexed real-time PCR can be carried out, i.e. there is a limit to the number
20 of different polynucleotides that may be amplified in a single reaction.

There is therefore a need for an improved method for monitoring the amplification process. In particular, there is a need for a method which can be used to carry out multiplexed reactions in an automated process.

Summary of the Invention

25 The present invention is based on the realisation that the progress of an amplification reaction may be monitored by detecting the interaction between an amplified product and a molecule that has the capability of reacting with the amplified product.

According to a first aspect of the present invention, a method for
30 monitoring a polynucleotide amplification reaction comprises the steps of:

- (i) carrying out a reaction for the amplification of a target polynucleotide;

(ii) either during or after the amplification reaction contacting the amplified product with a molecule that binds to or interacts with a polynucleotide; and

(iii) detecting the interaction between the amplified product and the molecule by measuring changes in applied radiation,

5 wherein the molecule is immobilised to a support material.

The method of the invention can be carried out without the requirement for fluorophores and therefore overcomes the disadvantages associated with the use of fluorophores. In addition, the method of the invention can be carried out 10 on a real-time basis, without the need to obtain samples during the amplification process. Real time multiplexed monitoring of an amplification reaction can therefore be achieved.

Description of the Invention

The present invention provides a way of monitoring the progress of a 15 polynucleotide amplification reaction involving the analysis of the interaction between an amplified polynucleotide and a molecule that interacts with or binds to a polynucleotide.

The term "polynucleotide" as used herein is to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other 20 hybridising nucleic acid-like molecules, e.g. peptide nucleic acid (PNA). The term encompasses oligonucleotides which comprise short sequences of nucleic acid monomers.

The present invention relies on the use of a molecule that binds to or otherwise interacts with a polynucleotide. The molecule may be any molecule 25 that binds to a polynucleotide in a specific or non-specific manner. The molecule may interact with a polynucleotide which is in a double stranded or single stranded form. Molecules which interact with polynucleotides would be apparent to the skilled person. In one embodiment, the molecule is a protein and may be a DNA or RNA binding protein. Suitable proteins may be recombinant proteins 30 which have been modified to contain a site-specific polynucleotide binding domain. Such domains are well known in the art, and are disclosed in Duncan et al., Genes Dev., 1994; 8(4): 465-80. Examples of proteins which interact with

polynucleotides and which are therefore within the scope of the present invention include: helicases, transcriptases, primases and histones.

In a particularly preferred embodiment, the molecule is a polymerase enzyme which may be utilised in the amplification reaction. Accordingly, the
5 detection of the interaction may be carried out at the same time as the amplification reaction proceeds.

In a further embodiment, the molecule is a single stranded polynucleotide having a sequence that is complementary to at least part of the amplified polynucleotide. In this embodiment, a plurality of such polynucleotides will be
10 immobilised on a support material and hybridisation of the amplified polynucleotides onto the immobilised polynucleotides can be monitored by monitoring the change in applied radiation which occurs on hybridisation.

It will be usual to utilise a plurality of immobilised molecules, so that multiple interactions can be detected. It is preferable to include an excess of
15 immobilised molecules compared to amplified product, as this will ensure that there is the possibility that all amplified products can interact with a molecule.

Techniques for immobilising molecules of support materials are known to those skilled in the art. Suitable support materials will also be known, and the choice suitable material may depend on the detection technique, as many
20 techniques that monitor changes in electromagnetic radiation require specific substrates.

The detection of the interaction between an amplified product and the molecule is carried out by measuring changes in applied radiation. Measuring the changes in radiation occurs on interaction between an amplified product and
25 the molecule may be carried out using conventional apparatus. In a preferred embodiment, changes in radiation are monitored by utilising surface electromagnetic wave technology.

Biosensors incorporating surface electromagnetic wave technology (and, in particular, surface plasmon resonance - SPR - sensors) are based on the
30 sensitivity of surface electromagnetic waves (SEW) to the refractive index of the thin layer adjacent to the surface where the SEW propagates. In the biosensor the amplified products are allowed to flow across the surface containing

immobilised molecule. As binding occurs, the accumulation or redistribution of mass on the surface changes the local refractive index that can be monitored in real time by the sensor.

Several methods of SPR registration have been proposed and realised

5 in biosensors. The most popular methods are based on the Kretschmann-Raether configuration where intensity of the light reflected from sensor is monitored. This technique, considered to be one of the most sensitive, is described in J. Homola et al, Sensors and Actuators B 54, p.3-15 (1999) and has a detection limit of 5×10^{-7} refractive index units. Measuring SPR phase changes

10 can further increase the sensitivity of the sensor by one or two orders of magnitude. This is described in Nelson et al, Sensors and Actuators B 35-36, p. 187 (1996) and in Kabashkin et al, Optics Communications 150, p.5 (1998). Prior art interferometric devices such as a Mach Zehnder device have been configured to measure variations in the refractive index at the sensor surface via

15 phase shifts. This is disclosed in WO01/20295. The configuration requires four independent components and is sensitive to sub-wavelength relative replacements of these components and hence very small mechanical and environment perturbations. A mechanically more robust monolithic interferometric design is outlined in WO03014715.

20 In a preferred embodiment, a surface electromagnetic wave (SEW) sensor system is used which can compensate for changes in the bulk refractive index of a buffer or which allows the contribution of the bulk refractive index to an interference pattern to be separated from the contribution of an analyte absorbed on the sensor surface. The biosensor therefore comprises:

25 a coherent radiation source for producing an incident wave;

 a carrier surface for supporting an immobilised molecule, the carrier surface counted on a substrate and capable of supporting surface electromagnetic waves (SEW);

 means for splitting the incident wave into an SEW and a first scattered

30 wave, wherein the SEW propagates along the carrier surface and interacts with the immobilised molecule;

 means for generating a second scattered wave from the SEW; and,

a detector for monitoring the interference between the first scattered wave and the second scattered wave.

In this embodiment, a coherent optical beam generated by a monochromatic laser is focussed using a lens, onto the edge of a metallic film able to support surface electromagnetic waves (SEWs). The optical beam passes through a glass prism on which the metallic film is mounted. A near-infrared laser is used as the illumination source. Using a near-infrared source has the advantage of long propagation length for surface plasmons in gold and silver while conventional optics can be still used for imaging and illumination.

10 However, other monochromatic sources are suitable and may be used.

The laser provides a p-polarised beam. The p-polarised laser beam passes through a focussing lens and then through the glass prism on which a substrate with a microfabricated metal film (structure) is attached, using an index matching liquid or gel in a fluidic cell. The index matching gel reduces light scattering and creates a continuous optical path. The glass prism may be triangular prism or a hemi-cylindrical prism. The laser beam falls on the glass/liquid interface at an incidence angle larger than the angle of total internal reflection, so that the laser beam totally reflected except at a small area around the edge of the metal structure. At the edge of the structure the evanescent light wave formed on reflection is partly scattered into light propagating through the fluidic cell and partly scattered into a plasmon wave propagating along the metal structure. The plasmon wave is further scattered by the structure to produce light waves. Waves propagate through the liquid cell and produce an interference fringe pattern on a measurement device.

25 The metal structure can be formed from gold or silver, or any other metal capable of supporting surface plasmons or a combination of them, or alternatively a dielectric multilayer supporting a SEW. It is preferred to use either gold or silver/gold multilayer to increase surface plasmon propagation length. The metal structure can be deposited on the prism using a lithographic process.

30 Adaptations of this technique are described in co-pending international patent application PCT/GB03/03803, the content of which is hereby incorporated by reference.

The amplification reaction is carried out using conventional reagents and conditions. In summary, a target polynucleotide is contacted with a polymerase enzyme, the necessary primer molecules and the various nucleic acid monomers (bases) so that incorporation of the monomers onto the target polynucleotide can occur. A polynucleotide complementary to that of the target polynucleotide is then synthesised by the polymerase. After the complementary polynucleotide is synthesised the temperature at which the reaction is performed increased so that the hybridisation between the complement and the target is disrupted and dissociation occurs. The target and the complement may then be used as substrates for further amplification.

In a preferred embodiment, the polymerase reaction is carried out within a sealed micro-flow cell. The reactants are introduced into the flow cell which is then sealed by closing input and output valves. An integrated pump may be incorporated to maintain the reaction/PCR fluid flowing in the closed cell. The reactant mixture is allowed to flow over the immobilised molecule so that amplified products can interact with the molecule, permitting the detection of the interaction and consequently the quantification of the amplification process. As the amplification reaction proceeds the increased amount of amplified product will interact with additional immobilised molecules, generating an increase in the signal detected. Defecting the signal permits the amplification reaction to be quantified.

Multiplexed reactions may be carried out by incorporating spatially separated molecules which interact specifically with one type of amplification product. For example, DNA binding proteins may be used which contain different polynucleotide binding domains. It is therefore possible to distinguish the amplification products based on their interaction with different binding proteins. Alternatively, if the polynucleotides are used as the binding molecule, they can be designed so that a range of different sequences are present at defined locations, allowing sequence specific interactions to be monitored.